Effect of hypoxia and dexamethasone on inflammation and ion transporter function in pulmonary cells

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Abstract: Dexamethasone has been found to reduce the incidence of high-altitude pulmonary oedema. Mechanisms explaining this effect still remain unclear. We assessed the effect of dexamethasone using established cell lines, including rat alveolar epithelial cells (AEC), pulmonary artery endothelial cells (RPAEC) and alveolar macrophages (MAC), in an environment of low oxygen, simulating a condition of alveolar hypoxia as found at high altitude. Inflammatory mediators and ion transporter expression were quantified. Based on earlier results, we hypothesized that hypoxic conditions trigger inflammation. AEC, RPAEC and MAC, pre-incubated for 1 h with or without dexamethasone (10(-7) mol/l), were subsequently exposed to mild hypoxia (5% O(2), or normoxia as control) for 24 h. mRNA and protein levels of cytokine-induced neutrophil chemoattractant-1, monocyte chemoattractant protein-1 and interleukin-6 were analysed. mRNA expression and functional activity of the apical epithelial sodium channel and basolateral Na(+)/K(+)-ATPase were determined using radioactive marker ions. In all three types of pulmonary cells hypoxic conditions led to an attenuated secretion of inflammatory mediators, which was even more pronounced in dexamethasone pretreated samples. Function of Na(+)/K(+)-ATPase was not significantly influenced by hypoxia or dexamethasone, while activity of epithelial sodium channels was decreased under hypoxic conditions. When pre-incubated with dexamethasone, however, transporter activity was partially maintained. These findings illustrate that long-term hypoxia does not trigger an inflammatory response. The ion transport across apical epithelial sodium channels under hypoxic conditions is ameliorated in cells treated with dexamethasone.

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Effect of Hypoxia and Dexamethasone on Inflammation and Ion Transporter Function in Pulmonary Cells

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Abstract

Dexamethasone has been found to reduce the incidence of high altitude pulmonary edema. Mechanisms explaining this effect still remain unclear. We assessed the effect of dexamethasone using established cell lines including rat alveolar epithelial cells (AEC), pulmonary artery endothelial cells (RPAEC) and alveolar macrophages (MAC) in an environment of low oxygen, simulating a condition of alveolar hypoxia as found at high altitude. Inflammatory mediators and ion transporter expression were quantified. Based on earlier results we hypothesized that hypoxic conditions trigger inflammation.

AEC, RPAEC and MAC, preincubated for 1 hour with or without dexamethasone (10^{-7} mol/l), were subsequently exposed to mild hypoxia (5% O_2, or normoxia as control) for 24 hours. mRNA and protein levels of cytokine-induced neutrophil chemoattractant-1, monocyte chemotactic protein-1 and interleukin-6 were analyzed. mRNA expression and functional activity of the apical epithelial sodium channel and basolateral Na+/K+-ATPase were determined using radioactive marker ions.

In all three types of pulmonary cells hypoxic conditions lead to an attenuated secretion of inflammatory mediators, which was even more pronounced in dexamethasone pretreated samples. Function of Na+/K+-ATPase was not significantly influenced by hypoxia or dexamethasone, while activity of epithelial sodium channel was decreased under hypoxic conditions. When preincubated with dexamethasone, however, transporter activity was partially maintained.

These findings illustrate that long term hypoxia does not trigger an inflammatory response. The ion transport across apical epithelial sodium channels under hypoxic conditions is ameliorated in cells treated with dexamethasone.

Introduction

Alveolar hypoxia has been shown to induce injury within the lung parenchyma including epithelial lung damage, capillary leakage and edema formation [1]. Conditions potentially imparting states of alveolar hypoxia include exposure to high altitudes, lung diseases, bulbar cerebral injuries, and overdoses of narcotic agents (alcohols, barbiturates, opioids).

In particular, high altitude pulmonary edema (HAPE) is a high permeability pulmonary edema caused by increased pulmonary capillary pressure leading to a protein rich edema fluid [2]. Elevated pulmonary capillary pressure is most likely induced by hypoxic pulmonary vasoconstriction. Pulmonary vasodilators are efficient in treating and preventing HAPE, suggesting that elevated pulmonary artery pressure is indeed a crucial pathophysiologic mechanism [3, 4]. Administration of corticosteroids such as dexamethasone has been found to reduce the incidence of HAPE [5, 6]. Mechanisms explaining this effect are still not thoroughly clear: Besides a decrease of the systolic pulmonary artery pressure, an amelioration of hypoxia-induced impairment of fluid clearance has been suggested as underlying mechanism of the dexamethasone effect [6]. Moreover, there is a known crosslink between hypoxia and inflammation pathways [7]. Proinflammatory proteins such as interleukin-6 (IL-6) and C-reactive protein (CRP) have been found to be increased in response to high altitude exposure [8]. Attenuation in proinflammatory cytokine expression by corticosteroids might therefore also be an underlying factor for the preventing effect of dexamethasone.

In inflammatory processes, chemokines such as cytokine-induce neutrophil chemoattractant protein-1 CINC-1) [9], monocyte chemotactic protein-1 (MCP-1) [10] and IL-6 [11] are among the most important inflammatory mediators directly or indirectly orchestrating the effector cell migration to the location of tissue injury, where an elevated expression of adhesion molecules including intercellular adhesion molecules (ICAM-1) mediate effector-target cell interaction in the respiratory and vascular compartment of the lung [12, 13] (**Figure**

1), an interplay between alveolar epithelial (AEC), endothelial cells (RPAEC) and alveolar macrophages (MAC).

In this study, we hypothesized that dexamethasone treatment before and during exposure to hypoxic conditions attenuates inflammatory mediator synthesis in AEC, RPAEC and MAC *in vitro* and enhances alveolar water clearance in AEC. The study aims at a better understanding of the effect of dexamethasone on alveolar water clearance and the secretion of proinflammatory mediators under long-term hypoxic conditions as found in high altitudes. Insights regarding the effect of dexamethasone in alveolar hypoxia may have implications on the future treatment of HAPE.

Material & Methods

Experimental design

Conditions at high altitude were simulated exposing AEC, RPAEC as well as MAC to 5% hypoxia during 24h. After preincubation with either dexamethasone or NaCl containing cell culture medium for 1 hour, cells were exposed for 24 hours to hypoxia or normoxia in a cell incubator (Bioblock, Ittigen, Switzerland) with adjustable O_2 levels. Oxygen concentration was reached by flushing nitrogen through the exposure chamber and continuously monitored by an oxygen sensor. During hypoxic conditions, oxygen and carbon dioxide concentrations were both 5%, whereas under normoxic control conditions, oxygen was 21% and CO_2 was 5%. Temperature was kept constant at 37°C during all experiments. After 24 hour-exposure to the respective atmosphere, supernatants and or cells were immediately collected without reoxygenation.

Alveolar epithelial cells (L2)

The L2 cell line (CCL 149; American Type Culture Collection, Rockville, MD, USA) was used as representatives of rat alveolar epithelial cells [14]. They were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen AG, Basel, Switzerland), enriched with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were grown in uncoated 35x10 mm plates (Corning Inc., Corning, NY, USA) to a confluent monolayer. Before performing the experiments, the medium was changed to DMEM/1% FBS.

Rat pulmonary artery endothelial cells (RPAEC)

Rat pulmonary artery endothelial cells, kindly provided by Dr. Roscoe Warner, (Department of Pathology, University of Michigan, Ann Arbor, MI, USA), were cultured in Dulbecco's modified eagle medium (DMEM) with 10% FBS, 1% penicillin–streptomycin and 1% HEPES. Prior to the experiments, medium was changed to DMEM/1% FBS.

Rat alveolar macrophages (MAC)

The rat alveolar macrophage cell line CRL-2192 was obtained from the American Type Culture Collection (Manassas, VA, USA). Alveolar macrophages were cultured in nutrient mixture F-12Ham (Ham's F-12; Invitrogen Corporation, Carlsbad, CA, USA), completed with 15% FBS, 5% penicillin/streptomycin (10 000 U/l) (Invitrogen Corporation) and 5% HEPES (Invitrogen Corporation). The cells were grown to confluence. At day 3 cells were centrifuged for 3 min at 1250 rpm, and experiments were performed.

All three types of cells are represented by well established pulmonary cell lines.

Dexamethasone treatment

Dexamethasone (Mephamesone-4®, Mepha Pharma AG, Aesch, Switzerland) was dissolved in NaCl and diluted in culture medium DMEM/1%FBS to a concentration of 10^{-7} mol/l. In order to investigate the effect of dexamethasone on the secretion of inflammatory mediators and ion channel function under normoxic and hypoxic conditions, cells were pre-incubated (Bioblock, Ittigen, Switzerland) with dexamethasone-containing medium 1 hour before exposure to hypoxia/normoxia for 24 hours (NaCl served as negative control). Cells therefore underwent in total 25 hours of exposure to dexamethasone before measurements.

Cytotoxicity and viability tests

To determine possible cytotoxicity mediated by dexamethasone and/or hypoxia, we measured lactate dehydrogenase concentrations (LDH) (Promega, Madison, WI, USA) in cell supernatants. The cell's viability was monitoring by performing 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assays [15].

Enzyme linked immunoabsorbent assay (ELISA)

Sandwich ELISAs were performed according to the manufacturer's protocols. The concentration of the chemokines CINC-1 (R&D Systems Europe Ltd, Abingdon, UK), MCP-1 (BD Biosciences, San Diego, CA, USA), and the cytokine interleukin-6 (IL-6; R&D Systems Europe Ltd, Abingdon, UK) were measured. Endothelial cells did not express any IL-6 protein. Cell-based ICAM-1 ELISA was performed on AEC and RPAEC as previously described [16].

RNA-extraction, reverse transcriptase and real-time PCR

After collection of the supernatants, cell lysate was harvested using lysing buffer provided in the RNeasy® Mini Kit (Qiagen, Basel, Switzerland). RNA was isolated according to the manufacturer's protocol. After determination of RNA amount (NanoDrop ND 1000, NanoDrop Technologies, Delaware, USA), reverse transcription from RNA to cDNA was performed (GeneAmp 9700 system, Applied Biosystems, Branchburg, NJ, USA). The TaqMan Real-Time PCR System 7500 Fast (Applied Biosystems, Branchburg, NJ, USA) was used to amplify and simultaneously quantify the targeted molecules. Following primers were designed: CINC-1, MCP-1, IL-6, ICAM-1, α-ENaC and α-Na+/K+-ATPase [17]. Samples were normalized to the housekeeping gene 18S (all primers from Microsynth, Balgach, Switzerland; labelled TaqMan probes from Roche Applied Science, Switzerland; for details see **supporting information Table S8**).

Stimulation of AEC with dimethyloxallyl glycine (DMOG) instead of hypoxia

After preincubation with either dexamethasone or NaCl for 1 hour, AEC were incubated over a time period of 24 hours with DMOG (Sigma-Aldrich, Hamburg, Germany) at a concentration of 1mM [18] instead of hypoxia (5%).

^{22}Na influx studies

Sodium influx through ENaC was measured with the method established by Clerici et al [19]. Cells were rinsed twice and preincubated at 37°C for 20 min in buffered sodium-free medium. Thereafter cells were incubated with ^{22}Na for six minutes, and uptake was stopped by washing the cell monolayer. Events were detected by a liquid scintillation counter (Tri-carb 2900TR, Packard, IL, USA). To estimate the portion of ENaC in total ion flux ^{22}Na uptake inhibited by amiloride (100 µM; Sigma-Aldrich, Hamburg, Germany) was also measured.

^{86}Rubidium influx Studies

^{86}Rubidium (^{86}Rb), serving as surrogate for potassium [20], was used to quantify ion transport via Na+/K+-ATPase. Radioactive counts were measured using Tri-carb 2900TR, Packard, IL, USA. The portion of ^{86}Na uptake inhibited by Ouabain was also measured reflecting the portion of Na+/K+-ATPase in total ^{86}Na flux (4 mM; Sigma-Aldrich, Hamburg, Germany).

Statistics

Data are summarized as medians (quartiles). Matlab Software (MATLAB 2008R; Mathworks) and SPSS (SPSS Inc, Chicago, Ill) was used to perform all statistical analysis. Linear regression analysis was performed to assess the influences of hypoxia, dexamethasone and changes in viability / MTT (independent variables) on inflammatory mediator levels (dependent variable) (see **Table S2-S5** for the detailed statistic tables in the supporting information). Spearmans rank correlation analysis was performed to compare inflammatory mediator protein concentration to their related mRNA levels. Influence of hypoxia, dexamethasone treatment or viability / MTT (independent variables) on ion fluxes (dependent variable) was also analyzed using linear regression (see **Table S6-S7** for the detailed statistic tables in the supporting information). All experiments were at least performed three times each with four independent samples per group.

Results

Inflammatory response in AEC upon exposure hypoxic conditions

After 24 hours of exposure to hypoxic conditions both CINC-1 (-39%, p<0.001, R^2: 0.568) and MCP-1 (-42%, p < 0.001, R^2: 0.757) protein expressions were decreased (**Figure 2)**. This was not observed for IL-6 protein or ICAM-1 expression levels (**supporting information**, **Figure S1**), which remained unchanged. Dexamethasone incubation prior to hypoxia was found to decrease CINC-1 (-56%, p < 0.001, R^2: 0.568) and MCP-1 (-82%, p < 0.001, R^2: 0.787) protein levels even more compared to mere hypoxia treatment. Exposure to dexamethasone alone under normoxic conditions decreased CINC-1 (-32%, p < 0.001, R^2: 0.568), MCP-1 (-82%, p < 0.001, R^2: 0.757), and IL-6 protein expression (-36%, p = 0.004, R^2: 0.380). No changes in ICAM-1 protein levels were observed. Measured mRNA expression of CINC-1, MCP-1, and IL-6 (**Figure 2**) was in good correlation with observed inflammatory mediator protein levels (**supporting information, Table S1**), except for ICAM-1 where a decrease upon exposure to hypoxic conditions and dexamethasone was found on mRNA (-33%, p = 0.010, R2: 0.126), but not on protein level.

Inflammatory mediator expression in RPAEC after exposure to hypoxia

Inflammatory response upon hypoxic conditions and influence of dexamethasone was also evaluated in RPAEC mimicking the response of endothelial cells from the vascular compartment *in vitro* (**Figure 3**). In contrast to results from AEC, only a decrease in CINC-1 protein levels after 24 hours exposure to hypoxia was found (-35%, p < 0.001, R^2: 0.772). No significant changes were measured regarding mRNA levels of all cytokines, and protein expression of MCP-1 and ICAM-1. IL-6 protein was not detectable in supernatants of RPAEC. Similar to results from AEC, addition of dexamethasone attenuated MCP-1 protein and mRNA expression under normoxic (-47%, p < 0.001, R^2: 0.575) and hypoxic conditions

(-52%, $p < 0.001$, R^2: 0.575). ICAM-1 mRNA (- 44%, $p = 0.002$, R^2: 0.317), but not protein levels were slightly decreased after exposure to dexamethasone alone under normoxic conditions (**Figure 3**).

Inflammatory mediator expression in MAC after exposure to hypoxia

Effector cells response upon long-term incubation for 24 hours under hypoxic conditions of 5% oxygen was studied in alveolar macrophages. An attenuated expression of MCP-1 protein (-32%, $p < 0.001$, R^2: 0.851) and mRNA (-33%, $p = 0.031$, R^2: 0.524) was found after exposure to hypoxia (**Figure 4**). Addition of dexamethasone also provoked an even more pronounced decrease in MCP-1 protein levels (-91%, $p < 0.001$, R^2: 0.851). CINC-1 and IL-6 protein concentrations were below detection levels. Hypoxia attenuated ICAM-1 mRNA expression (-65%, $p<0.001$, R^2: 0.919). Dexamethasone incubation prior to hypoxia was found to decrease ICAM-1 (-80%, $p < 0.001$, R^2: 0.919) protein levels even more compared to mere hypoxia treatment.

Activation of hypoxic-inducible factor attenuates inflammatory mediator expression

Additional experiments in AEC were performed using DMOG to investigate whether activation of hypoxia-inducible factor-1α (HIF-1α) attenuates inflammatory mediator expression comparable to exposure to hypoxia (5% O_2). An attenuation of CINC-1 protein expression of -44% was measured after incubation with DMOG ($p < 0.001$, R^2: 0.934) (**Figure 5)**. In the DMOG and dexamethasone group CINC-1 protein expression was even more decreased (-59%, $p < 0.001$, R^2: 0.934). Stimulation with DMOG also attenuated MCP-1 protein secretion by -89% compared to control ($p < 0.001$; R^2: 0.863). Incubation of both dexamethasone and DMOG decreased MCP-1 protein by -80% ($p < 0.001$; R^2: 0.863). No differences were observed between samples incubated with DMOG and DMOG in the presence of dexamethasone.

Influence of hypoxia and dexamethasone on ion transporter expression and activity

To assess the influence of dexamethasone on sodium transport, mRNA expression of the basolateral sodium/potassium ATPase and of the apical epithelial sodium channels were measured (**Figure 1**). In addition, the influence of hypoxia and dexamethasone on inhibitor-sensitive (reflecting ENaC and Na+/K+-ATPase function) and inhibitor-insensitive portion of the total sodium transport was determined in ^{22}Na and ^{86}Rb influx studies. Total ^{22}Na flux was significantly decreased upon hypoxic conditions (-42%, $p < 0.001$, R^2: 0.415). When cells were preincubated with dexamethasone before exposure to hypoxic conditions, however, Na+ influx was partially maintained (-30% compared to control; $p = 0.001$; R^2: 0.415). Amiloride inhibited on average 45% of total ^{22}Na flux (-45%, $p < 0.001$, R^2: 0.415) which in turn reflects the portion of ENaC in total ^{22}Na flux. Amiloride-insensitive ^{22}Na flux was not significantly influenced by dexamethasone or hypoxia.

Preincubation with dexamethasone slightly increased mRNA expression of Na+/K+-ATPase in comparison to normoxic control after 24 hours (+36%, $p = 0.021$, R^2: 0.268). In experiments using ^{86}Rb as marker of ion flux, no increased Na+/K+-ATPase activity upon dexamethasone treatment was observed which is in contrast to results from mRNA assays. Addition of dexamethasone under normoxic condition even marginally decreased total ^{86}Rb flux (-14%, $p < 0.001$, R^2: 0.900). Ouabain impaired ^{86}Rubidium uptake on average by -68% ($p < 0.001$, R^2: 0.900) reflecting the portion of Na+/K+-ATPase in total ^{86}Rb flux. However, oubain-insensitive ^{86}Rubidium uptake was decreased by -10% in the presence of dexamethasone and hypoxia (-11%, $p = 0.017$, R2: 0.582).

Assessment of viability after exposure to hypoxic conditions and dexamethasone

Viability of the cells after exposure to hypoxic conditions and dexamethasone treatment was monitored by performing MTT assays (**Figure 7**). No relation between changes in MTT and inflammatory mediator expression was found for AEC, RPAEC or MAC. ^{86}Rb and ^{22}Na influx studies were not influenced by changes in MTT.

Discussion

With regard to the formation of hypoxia-induced pulmonary edema, the role of inflammation has been subject of numerous investigations, however, studies under isolated conditions (i.e. accurately controlled oxygen concentration, timing, and extent of reoxygenation) remain rare [8, 12, 21, 22]. In this study, we evaluated the inflammatory response in pulmonary epithelial and endothelial cells as well as in alveolar macrophages after 24 hours of hypoxia (5% oxygen; simulating conditions at high altitudes). Hypoxia induced a downregulation of inflammatory mediators, which was even more accentuated in the presence of dexamethasone. As marker of water flux, the activity of sodium transport in alveolar epithelial cells was determined. Under hypoxia, sodium ion transport was impaired in AEC, but transport activity of ENaC could be maintained when cells were pre-incubated with dexamethasone.

The influence of hypoxia on inflammation is discussed controversially in the literature: in previous studies, hypoxia has been shown to promote inflammation by activation of the inflammatory transcription factor NF-κβ, by prolonging neutrophil survival [23], and by induction of Toll-like receptors [24]. Raised levels of MCP-1 protein in macrophages were described in recent work of Chao and Madjdpour after exposure to conditions of low oxygen [25, 26]. Chao et al. demonstrated that alveolar macrophage-derived MCP-1 plays a crucial role in initiation of systemic inflammation under hypoxic conditions, where exposure of primary culture of rat alveolar macrophages to various oxygen concentrations (0%, 5%, 10%,

and 15%) induced a nearly 20-fold increase of MCP-1 expression. These macrophages, however, were exposed to hypoxia for only 30 min. In the present *in vitro* data we show that after an exposure of 24 hours mild hypoxia does not necessarily induce inflammation in pulmonary cells. In our study, an even attenuating effect of hypoxia was found for all three types of pulmonary cells. This was underlined by additional experiments using DMOG, in which also a downregulation of MCP-1 and CINC-1 protein expression has been found comparable to the experiments with hypoxia (5% oxygen). All these observations suggest that HIF-1α might mediate anti-inflammatory effects, which would be in good accordance with findings from previous studies [27-29]. However, from our data we cannot exclude that hypoxia may trigger inflammation at a very early phase [26]. Also further *in vitro* and *in vivo* studies under controlled conditions are required to ultimately clarify the role, mechanisms and dynamics of inflammation in hypoxia in general and the pathogenesis of hypoxia-induced edema.

Epithelial sodium channels have been identified as the major pathway for apical sodium entry in AEC thereby controlling fluid clearance from the alveolar space. In close interplay with apical sodium channels, basolateral Na+/K+-ATPase is a key player involved in transepithelial sodium transport by alveolar cells, and ensures efficient vectorial sodium transport [30, 31]. In the present study, 5% oxygen did not significantly influence mRNA expression of Na+/K+-ATPase and ENaC. Comparisons with other studies are delicate as exposure time, concentration of oxygen and cell types are different [32-34]. Because injury-induced changes in the mRNA expression of sodium transporters might not necessarily be in correlation with an impaired function, sodium transport in our experimental setting was additionally determined using radioactive marker ions. Sodium transport was significantly decreased in the presence of hypoxia, and was maintained when cells were pretreated with dexamethasone prior to hypoxic conditions. A similar observation was made in the work of Güney et al. [34]: In freshly prepared AEC of type II character the authors did not find a

difference in mRNA expression of ENaC in hypoxia or with dexamethasone exposure, while the impaired capacity of the epithelial sodium channel was positively influenced by dexamethasone [34]. A study from Mairbäurl et al. described an inhibition of the amiloride-sensitive portion of the active sodium transport to 55% upon exposure to 5% oxygen in primary cultured adult rat alveolar epithelial cell monolayers [35]. In our study mRNA expression of Na+/K+-ATPase was upregulated by dexamethasone. Activity, however, remained unchanged upon exposure to hypoxia and/or dexamethasone. Several factors might account for the differences in findings among different studies, whereas the concentration of oxygen (5% vs. 1.5%), exposure time (24 hours vs. 48 hours) and extent of reoxygenation might be of utmost importance.

Although numerous studies have investigated different aspects of hypoxia and high altitude pulmonary edema, it remains as hitherto controversial whether the inflammatory response upon alveolar hypoxia is just a secondary event to an edema of hydrostatic genesis (hypoxic pulmonary vasoconstriction; impaired fluid clearance due to decreased sodium and water transport [6]) or one of the causative factors in alveolar edema formation (such as in acute respiratory distress syndrome) [36]. The present study was performed with a special focus on *in vitro* evaluation of changes possibly observed in HAPE the findings might also play an important role in acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS). The role and time point of inflammation with regard to edema formation might tough not necessarily be the same, being aware of the substantial differences in pathogenesis of ALI/ARDS [37] and HAPE [22]. In conclusion, cells at the alveolar barrier do not seem to be involved in pro-inflammatory actions under prolonged hypoxic conditions. If the *in vitro* observed positive effect of dexamethasone of maintaining ENaC function in hypoxic conditions contributes to improved fluid reabsorption *in vivo* in situations such as HAPE remains to the subject of further investigations.

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Competing interest statement

The authors declare that they have no competing financial interests.

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Figure legends

Figure 1: Schematic drawing of an alveolus in state of early inflammation. *Effector cell recruitement by inflammatory mediators from pulmonary epithelial and endothelial cells is illustrated (left side). Water transport through ion channels located on the alveolar epithelial cell apical surface and baso-lateral membrane (right side) is impaired. Sodium enters the cell via apical epithelial sodium channels (ENaC). On the basolateral interface, sodium is excreted via Na+/K+-ATPase, while potassium enters the cell. Reabsoption of sodium goes along with the reabsorption of Cl^-, which generates an osmotic driving force for the transepithelial movement of water. Radioactive marker ions ($^{22}Na^+$ for Na^+ and ^{86}Rb for K^+) were used to measure the ion channel turnover. CINC-1=cytokine-induced neutrophil chemoattractant-1, MCP-1=monocyte chemoattractant protein-1, IL-6=interleukin-6.*

Figure 2: Alveolar epithelial cells. *Production of inflammatory mediators from alveolar epithelial cells type II (L2, AEC) under hypoxic and normoxic conditions, with or without dexamethasone pretreatment: Protein concentrations in supernatants (left) and mRNA expression levels (right). Hypoxia decreased CINC-1 and MCP-1 expression ($p<0.001$), but not IL-6 protein secretion ($p=0.08$). Addition of dexamethasone under hypoxic conditions attenuated CINC-1 and MCP-1 levels even more ($p<0.001$). CINC-1=cytokine-induced neutrophil chemoattractant-1, MCP-1=monocyte chemoattractant protein-1, IL-6=interleukin-6.*

Figure 3: Rat pulmonary artery endothelial cells. *Production of inflammatory mediators from rat pulmonary endothelial cells (RPAEC) under hypoxic and normoxic conditions, with or without dexamethasone pretreatment: Protein concentrations in supernatants (left) and mRNA expression levels (right). Hypoxia decreased CINC-1 ($p<0.001$), but not MCP-1 or*

6 protein expression. Dexamethasone attenuated MCP-1 secretion under normoxic and hypoxic conditions ($p<0.001$). CINC-1=cytokine-induced neutrophil chemoattractant-1, MCP-1=monocyte chemoattractant protein-1, ICAM-1=intercellular adhesion molecule-1.

Figure 4: Alveolar macrophages. *Production of inflammatory mediators from alveolar macrophages (MAC) under hypoxic and normoxic conditions, with or without dexamethasone pretreatment: both, hypoxic conditions and dexamethasone treatment decreased Monocyte Chemoattractant Protein-1 (MCP-1) protein concentration ($p<0.001$), as well as MCP-1 and Intercellular Adhesion Molecule-1 (ICAM-1) mRNA levels ($p<0.05$) in alveolar macrophages. Cytokine-induced neutrophil chemoattractant-1 and Interleukin-6 protein was not detectable in supernatants.*

Figure 5: Activation of hypoxic-inducible factor attenuates inflammatory mediator expression. *Similar as in experiments using exposure to hypoxia (5% oxygen), inflammatory mediator secretion is attenuated ($p<0.001$) in alveolar epithelial cells (AEC) stimulated with 1mM N-(Methoxyoxoacetyl)-glycine methyl ester (DMOG, an inhibitor of prolyl-4-hydroxylase). CINC-1=cytokine-induced neutrophil chemoattractant-1, MCP-1=monocyte chemoattractant protein-1.*

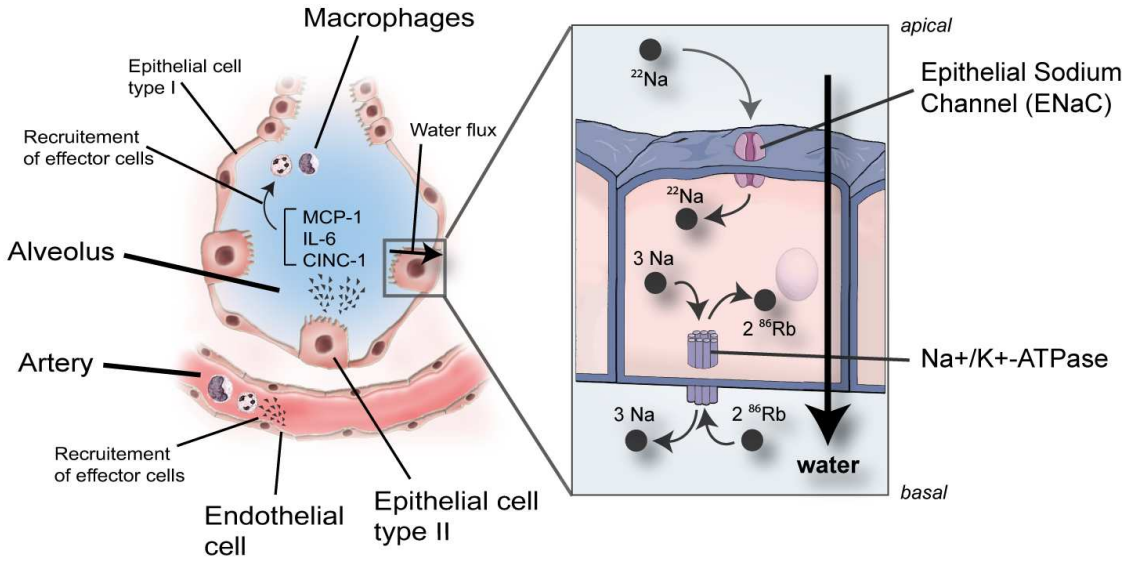
Figure 6: Ion channel expression and function. *Expression of apical epithelial sodium channel (αENaC) and basolateral Na+/K+-ATPase (mRNA-expression, left) with corresponding actual channel turnover measured by radioactive tracer ions (amiloride-sensitive ^{22}Na uptake for ENaC function, Ouabain-sensitive ^{86}Rubidium uptake for Na+/K+-ATPase function, right). Total ^{22}Na flux was decreased after exposure to hypoxia ($p<0.001$). When preincubated with dexamethasone ^{22}Na flux was partially maintained ($p=0.001$). In*

contrast, no increased ^{86}Rubidium uptake upon dexamethasone treatment was observed. Co=control, Hyp=hypoxia, Dex=dexamethasone

Figure 7: Viability. *Alveolar epithelial cells (AEC,) rat pulmonary artery endothelial cells (RPAEC), and alveolar macrophages (MAC) were pretreated with dexamethasone (or not) and exposed to 5% oxygen (or 21% as control) for 24 hours. MTT assays were performed: no influences of variations in viability on inflammatory mediator expression or ion fluxes were found.*

Figures

Figure 1: Schematic drawing of an alveolus in state of early inflammation.



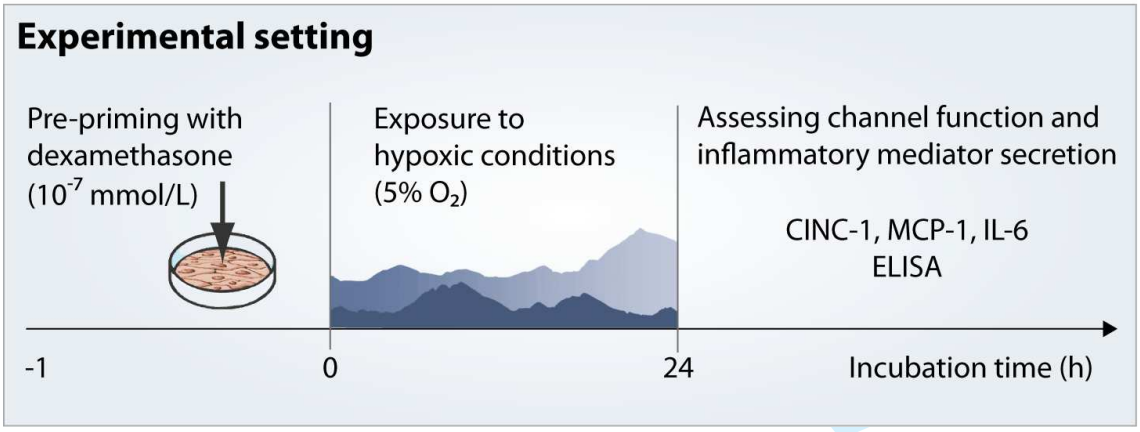


Figure 2: Alveolar epithelial cells.

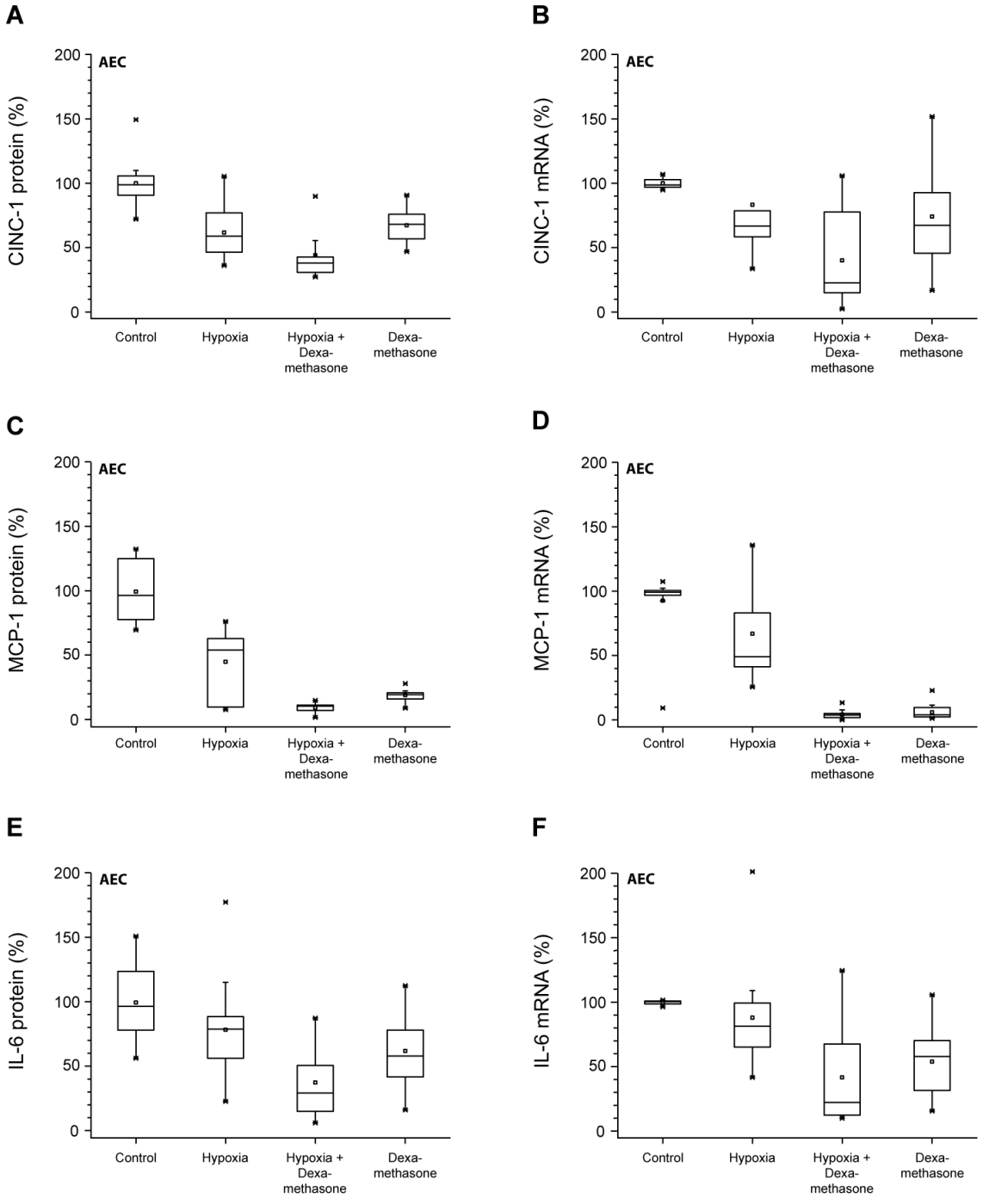


Figure 3: Rat pulmonary artery endothelial cells.

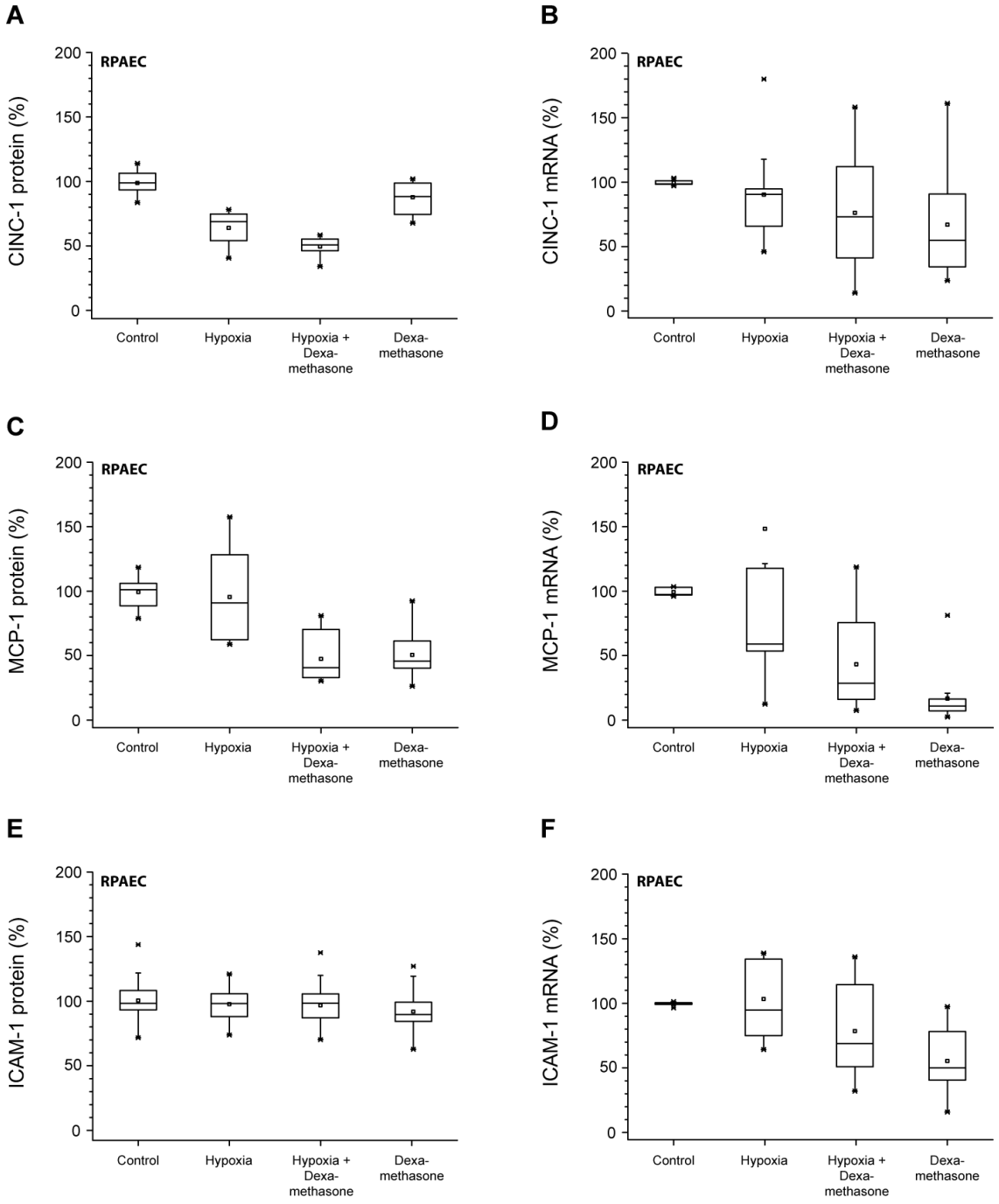


Figure 4: Alveolar macrophages.

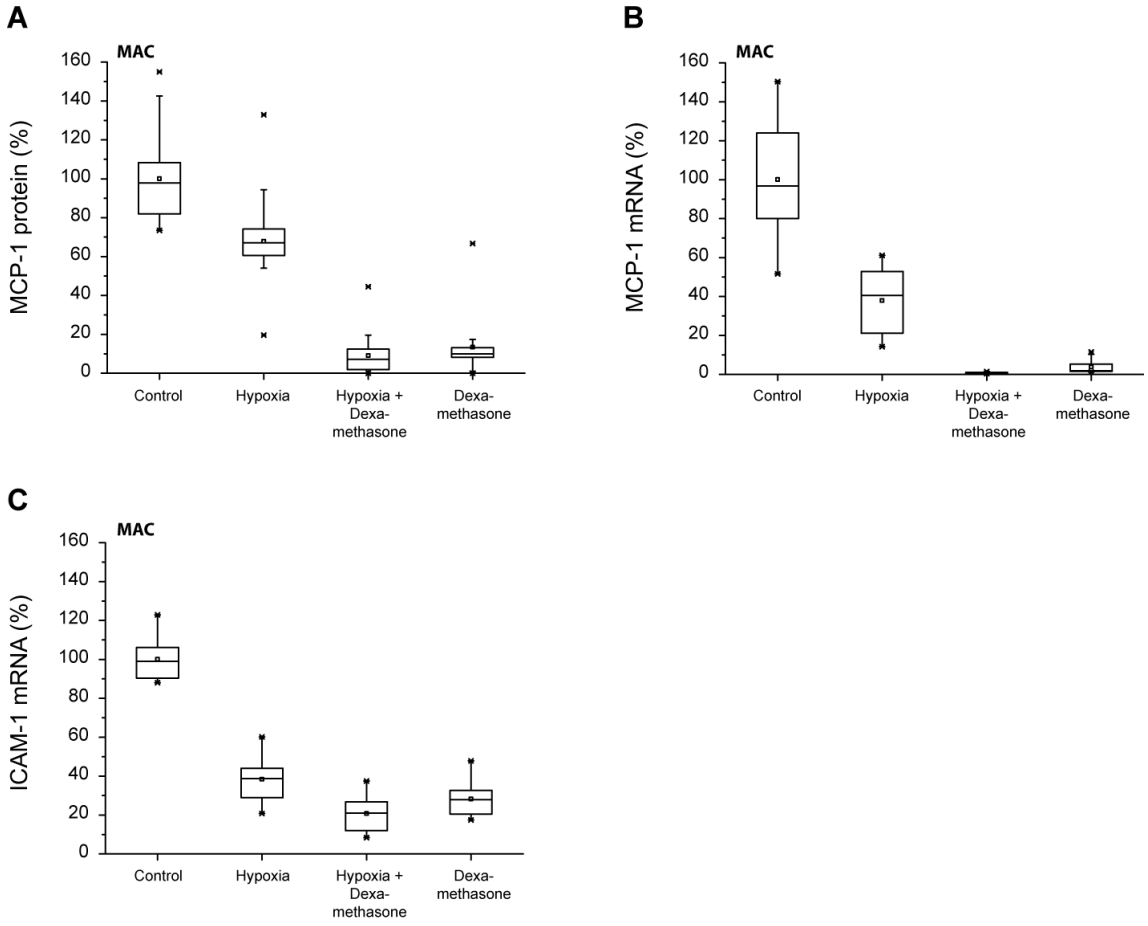


Figure 5: Activation of hypoxic-inducible factor attenuates inflammatory mediator expression.

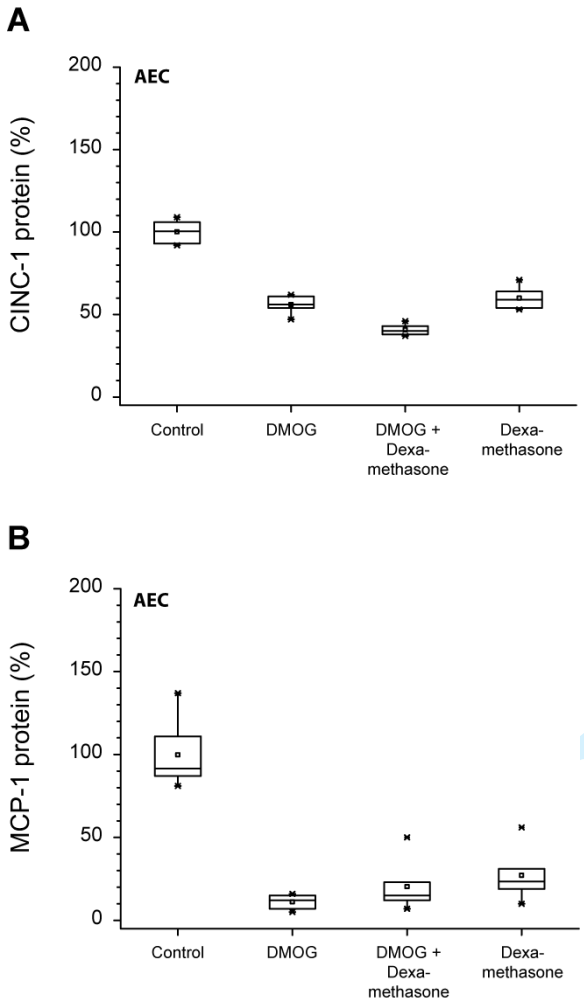


Figure 6: Ion channel expression and function.

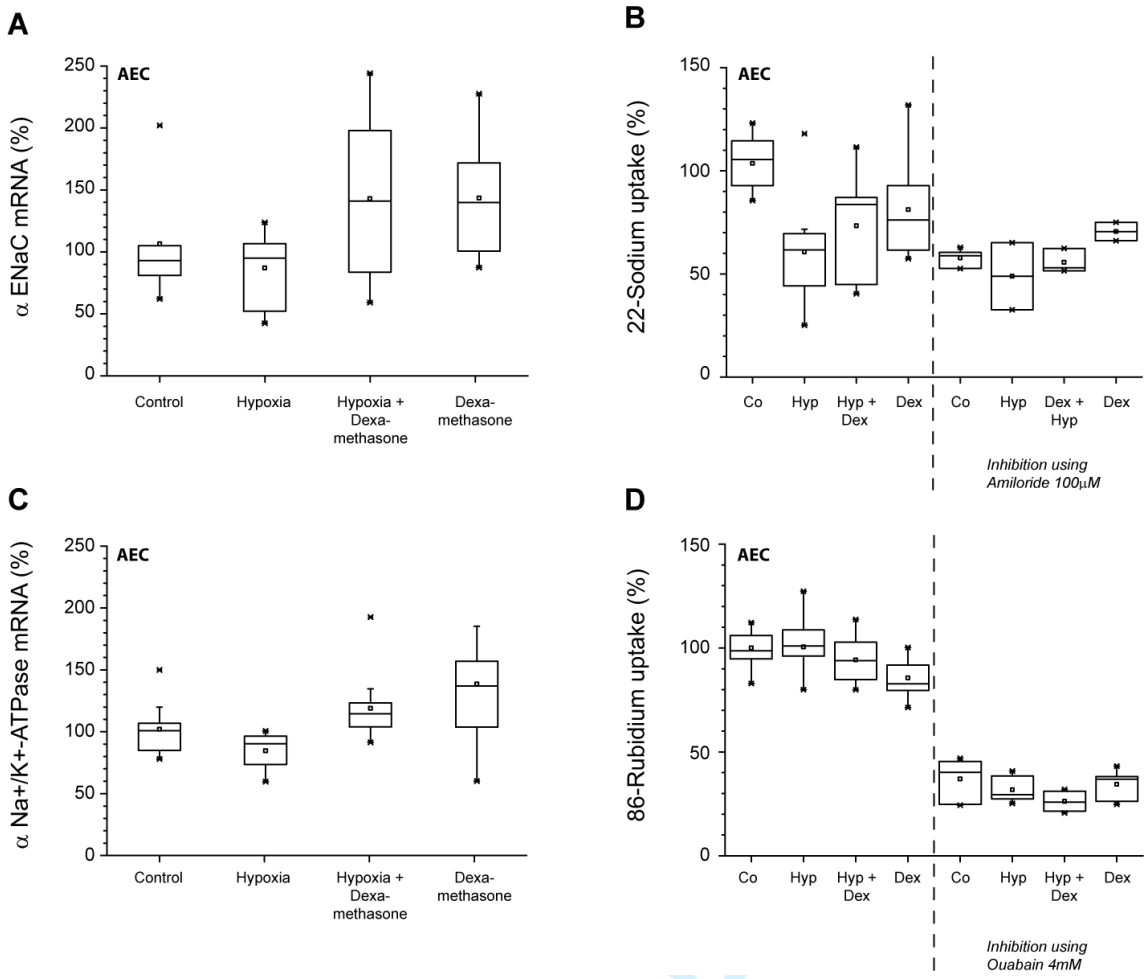
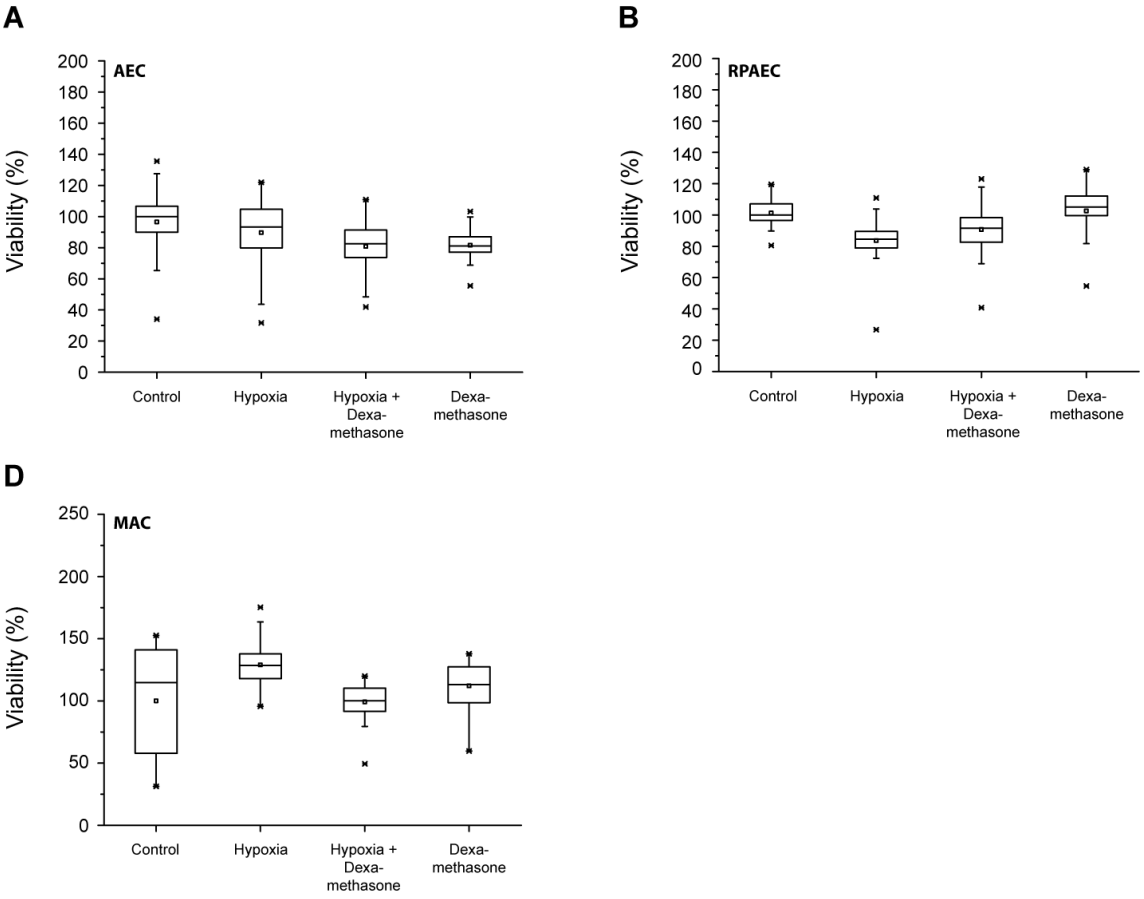


Figure 7: Viability.



Supporting Information

Effect of Hypoxia and Dexamethasone on Inflammation and Ion Channel Function in Lung Cells

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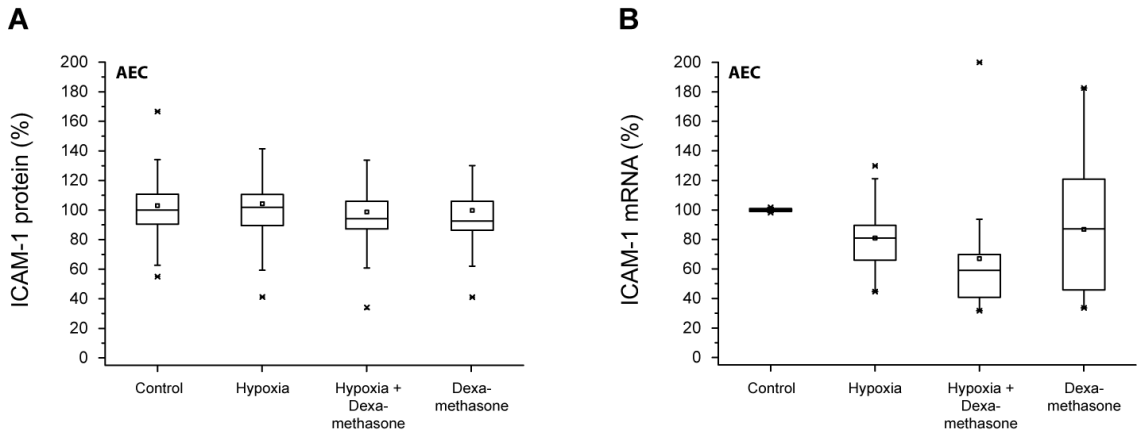

***Figure S1**: Intercellular adhesion molecule-1 (ICAM-1) expression in alveolar epithelial cells (AEC). AEC were pretreated with dexamethasone (or not) and exposed to 5% oxygen (or 21% as control) for 24 hours. Cell-based ELISA was performed (A) or mRNA determined (B).*

Table S1: TaqMan primers and probes

Gene	Primer sequence	Fragment size
CINC-1	forward: 5' cac act cca aca gag cac ca 3' reverse: 5' tga cag cgc acg tca ttg 3' probe #49	120bp
MCP-1	forward: 5' acg atc cac gtg gtg tct c 3' reverse: 5' gat cat ctt gcc agt gaa tga gt 3' probe #62	78bp
IL-6	forward: 5' ccc ttc agg aac agc tat gaa 3' reverse: 5' aca aca tca gtc cca aga agg 3' probe #20	74bp
ICAM-1	forward: 5' aac ctc agc ctc gct atg g 3' reverse: 5' act ttt gag ggg gac aca ga 3' probe #62	95bp
αENaC	forward: 5' tgt gac tac cga aag cag agc 3' reverse: 5' agg ctt ccg aca ctt gga g 3' probe #26	102bp
$\alpha Na^+/K^+$-ATPase	forward: 5' act tgg gca ctg aca tgg tt 3' reverse: 5' cac aag ttt gtc cgt ttt gg 3' probe #26	104bp
18S	forward: 5' gga gag gga gcc tga gaa ac 3' reverse: 5' tcg gga gtg ggt aat ttg c 3' probe #74	70bp

CINC-1 = cytokine-induced neutrophil chemoattractant-1; MCP-1 = monocyte chemoattractant protein-1; IL-6 = interleukin-6; ICAM-1 = intercellular adhesion molecule-1; αENaC = alpha subunit epithelial sodium channel

Table S2: Linear regression on expression of inflammatory mediator protein expression in alveolar epithelial cells (AEC)

CINC-1 = cytokine-induced neutrophil chemoattractant-1; MCP-1 = monocyte chemoattractant protein-1; IL-6 = interleukin-6; ICAM-1 = intercellular adhesion molecule-1; sign = significance; Hyp = hypoxia, Dex = dexamethasone

		Unstandardized Coefficients	95.0% Confidence Interval for B		Standardized Coefficients	Sign.	R^2	N
		B	Lower Bound	Upper Bound	Beta			
CINC-1	Hyp	-38.950	-53.645	-24.255	**-5.319**	**< .001** *	.568	57.000
	Hyp+Dex	-55.972	-69.697	-42.247	**-8.183**	**< .001** *		
	Dex	-32.392	-46.663	-18.121	**-4.555**	**< .001** *		
	Viability (MTT)	.007	-.308	.322	.045	.964		
MCP-1	Hyp	-42.049	-57.819	-26.280	**-.434**	**< .001** *	.757	72
	Hyp+Dex	-90.593	-104.611	-76.575	**-1.023**	**< .001** *		
	Dex	-82.096	-96.135	-68.057	**-.913**	**< .001** *		
	Viability (MTT)	.038	-.277	.352	.016	.811		
IL-6	Hyp	-22.051	-46.827	2.724	-.249	.080	.380	57.000
	Hyp+Dex	-61.781	-84.920	-38.641	**-.762**	**< .001** *		
	Dex	-36.450	-60.510	-12.390	**-.433**	**.004** *		
	Viability (MTT)	.152	-.379	.683	.066	.568		
ICAM-1	Hyp	4.368	-18.432	27.167	.068	.702	.076	53
	Hyp+Dex	7.766	-14.825	30.358	.120	.493		
	Dex	-11.404	-33.547	10.739	-.185	.306		
	Viability (MTT)	.039	-.454	.532	.023	.874		

Table S3: Linear regression on expression of inflammatory mediator protein expression in rat pulmonary artery endothelial cells (RPAEC)

CINC-1 = cytokine-induced neutrophil chemoattractant-1; MCP-1 = monocyte chemoattractant protein-1; IL-6 = interleukin-6; ICAM-1 = intercellular adhesion molecule-1; sign = significance; Hyp = hypoxia, Dex = dexamethasone

		Unstandardized Coefficients	95.0% Confidence Interval for B		Standardized Coefficients	Sign.	R^2	N
		B	Lower Bound	Upper Bound	Beta			
CINC-1	Hyp	-35.456	-48.336	-22.576	**-.730**	**< .001** *	0.772	38
	Hyp+Dex	-46.665	-57.129	-36.201	**-.961**	**< .001** *		
	Dex	-10.612	-21.796	.572	-.233	.062		
	Viability (MTT)	-.036	-.468	.396	-.025	.866		
MCP-1	Hyp	-5.529	-27.716	16.657	-.070	.619	0.575	55
	Hyp+Dex	-52.210	-68.992	-35.428	**-.731**	**< .001** *		
	Dex	-47.483	-66.529	-28.437	**-.678**	**< .001** *		
	Viability (MTT)	-.089	-.775	.598	-.040	.796		
IL-6	Hyp Hyp+Dex Dex Viability (MTT)	*Values below detection limit of the ELISA*						
ICAM-1	Hyp	4.781	-8.300	17.863	.186	.463	0.095	40
	Hyp+Dex	3.268	-7.209	13.745	.136	.531		
	Dex	-.426	-11.838	10.987	-.018	.940		
	Viability (MTT)	-.095	-.524	.333	-.125	.654		

Table S4: Linear regression on expression of inflammatory mediator protein expression in alveolar macrophages (MAC)

CINC-1 = cytokine-induced neutrophil chemoattractant-1; MCP-1 = monocyte chemoattractant protein-1; IL-6 = interleukin-6; ICAM-1 = intercellular adhesion molecule-1; sign = significance; Hyp = hypoxia, Dex = dexamethasone

		Unstandardized Coefficients	95.0% Confidence Interval for B		Standardized Coefficients	Sign.	R^2	N
		B	Lower Bound	Upper Bound	Beta			
CINC-1	Hyp Hyp+Dex Dex Viability (MTT)	*Values below detection limit of the ELISA*						
MCP-1	Hyp	-31.537	-39.107	-23.968	**-.357**	**< .001** *	0.851	140
	Hyp+Dex	-90.844	-98.354	-83.334	**-1.027**	**< .001** *		
	Dex	-87.533	-95.857	-79.209	**-.870**	**< .001** *		
	Viability (MTT)	-10.073	-24.929	4.782	-.046	.182		
IL-6	Hyp Hyp+Dex Dex Viability (MTT)	*Values below detection limit of the ELISA*						
ICAM-1	Hyp Hyp+Dex Dex Viability (MTT)	*Cell-based ELISA not applicable (adherence is a prerequisite)*						

Table S5: Linear regression on expression of inflammatory mediator expression after stimulation with DMOG

CINC-1 = cytokine-induced neutrophil chemoattractant-1; MCP-1 = monocyte chemoattractant protein-1; DMOG = N-(Methoxyoxoacetyl)-glycine methyl ester
B = beta coefficient; sign = significance

CINC-1 protein

Independent variables	**Unstandardized Coefficients**	**95.0% Confidence Interval for B**		**Standardized Coefficients**	**Sign.**
	B	Lower Bound	Upper Bound	Beta	
DMOG	-44.167	-51.313	-37.021	-.845	**< .001 ***
DMOG + Dexamethasone	-59.500	-66.646	-52.354	-1.138	**< .001 ***
Dexamethasone	-40.167	-47.313	-33.021	-.768	**< .001 ***

R^2: 0.934; N=24; dependent variable: CINC-1 protein

MCP-1 protein

Independent variables	**Unstandardized Coefficients**	**95.0% Confidence Interval for B**		**Standardized Coefficients**	**Sign.**
	B	Lower Bound	Upper Bound	Beta	
DMOG	-1217.167	-1470.948	-963.385	-1.013	**< .001 ***
DMOG + Dexamethasone	-1092.167	-1345.948	-838.385	-.909	**< .001 ***
Dexamethasone	-998.000	-1251.781	-744.219	-.830	**< .001 ***

R^2: 0.863; N=24; dependent variable: MCP-1 protein

Table S6: Linear regression regarding influence of hypoxia and dexamethasone on ^{22}sodium uptake

Hyp = hypoxia, Dex = dexamethasone; sign = significance; dependent variable: ^{22} sodium uptake

	Unstandardized Coefficients	95.0% Confidence Interval for B		Standardized Coefficients	Sign.	R^2	N
	B	Lower Bound	Upper Bound	Beta			
Hyp	-41.790	-60.044	-23.537	**-.660**	**< .001** *		
Hyp+Dex	-29.838	-47.192	-12.483	**-.487**	**.001** *		
Dex	-22.517	-39.744	-5.289	**-.368**	**.011** *	0.415	58
Viability (MTT)	-.077	-.409	.256	-.052	.645		
Amiloride-sensitivity	-45.141	-62.290	-27.991	**-.759**	**< .001** *		

Influences of dexamethasone / hypoxia on amiloride-insensitive ^{22} sodium uptake

	Unstandardized Coefficients	95.0% Confidence Interval for B		Standardized Coefficients	Sign.	R^2	N
	B	Lower Bound	Upper Bound	Beta			
Hyp + Amiloride	-9.395	-27.552	8.762	-.349	.267		
Hyp+Dex + Amiloride	-2.181	-17.614	13.251	-.095	.753	0.645	15
Dex + Amiloride	13.006	-4.841	30.854	.483	.131		
Viability (MTT)	-.063	-.448	.322	-.105	.714		

Table S7: Linear regression regarding influence of hypoxia and dexamethasone on ^{86}rubidium uptake

Hyp = hypoxia, Dex = dexamethasone; sign = significance; dependent variable: ^{86} rubidium uptake

	Unstandardized Coefficients	95.0% Confidence Interval for B		Standardized Coefficients	Sign.	R^2	N
	B	Lower Bound	Upper Bound	Beta			
Hyp	.446	-6.078	6.971	.006	.892		
Hyp+Dex	-5.684	-12.026	.658	-.076	.078		
Dex	-14.415	-20.858	-7.972	**-.188**	**< .001 ***	0.900	95
Viability (MTT)	.009	-.107	.124	.005	.884		
Ouabain-sensitivity	-67.760	-73.771	-61.749	**-1.004**	**< .001 ***		

Influences of dexamethasone / hypoxia on ouabain-insensitive ^{86} rubidium uptake

	Unstandardized Coefficients	95.0% Confidence Interval for B		Standardized Coefficients	Sign.	R^2	N
	B	Lower Bound	Upper Bound	Beta			
Hyp + Ouabain	-6.508	-15.335	2.319	-.360	.139		
Hyp+Dex + Ouabain	-10.756	-19.395	-2.117	-.595	.017	0.582	24
Dex + Ouabain	-.883	-9.861	8.096	-.049	.839		
Viability (MTT)	.147	-.059	.352	.311	.151		

Table S8: *Correlation analysis on mRNA expression and measured inflammatory mediator protein levels in alveolar epithelial cells (AEC).*

	Correlation Coefficient	**Sig. (2-tailed)**	**N**
CINC-1	.448	5.307E-04 **	56
MCP-1	.564	4.0145E-06 **	58
IL-6	.280	3.654E-02 *	56
ICAM-1	-0.134	0.328	55

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

CINC-1 = cytokine-induced neutrophil chemoattractant-1; MCP-1 = monocyte chemoattractant protein-1; IL-6 = interleukin-6; ICAM-1 = intercellular adhesion molecule-1; sig = significance